THE ISOLATION AND IDENTIFICATION OF PROBABLE FOOD POISONING STAPHYLOCOCCI FROM MILK

by

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INTRODUCTION

Staphylococci, as an etiological agent for food poisoning, have been a factor for bacteriologists to consider since 1914. Most of the food poisoning outbreaks due to staphylococci have been caused by the presence of these organisms in milk and milk products. Since staphylococci are common in all samples of milk it would be of value to determine a method to differentiate the food poisoning from the non-food poisoning types. Streptococcus mastitis in dairy cattle is very common. It seemed desirable, therefore, to undertake a study to determine the percentage of staphylococci of the probable food poisoning type that would be found in milk in animals of Kansas dairy herds and to ascertain the correlation between probable food poisoning staphylococci occurring in animals suffering from chronic mastitis caused by Streptococcus agalactiae and those animals free of such infections. Among other reasons for making the study were: (1) the fact that public health officials appear to be concerned with the detection and elimination from dairy herds only those animals which are suffering from mastitis causedby various types of streptococci; (2) because evidence is accumulating that shows the importance of staphylococci as food poisoning agents in milk and other dairy products, and (3) because staphylococci were constantly observed in blood agar plates which were negative for streptococci in routine examinations. At the present time the only animals that are

usually eliminated from a herd are those from which long chained streptococci can be demonstrated in the milk.

REVIEW OF LITERATURE

The first case of food poisoning by staphylococci was recorded by Barber (1914) who reported on a series of cases of acute gastroenteritis which was caused by a white staphylococci found in milk. No other cases of food poisoning were reported in the literature until Dack, Cary, Woolpert and Wiggers (1930) found a case due to a yellow staphylococcus.

Food poisoning organisms from these first cases were tested by feeding experiments on human beings and monkeys. Since feeding experiments of this type would be impossible in routine examinations, *in vitro* tests have been developed for the identification of these organisms. Stone (1935) described a gelatin medium (later modified by the addition of agar) in which the food poisoning strains utilized the gelatin, while the non-food poisoning strains did not. Chapman, Lieb, and Curcio (1937) advocated the most comprehensive *in vitro* tests: (1) pigment production, (2) hemolysis production, (3) coagulase production, (4) color of growth on crystal violet agar, (5) luxuriance of growth on brom thymol blue agar, (6) fermentation of mannitol on phenol red mannitol agar, and (7) liquefaction of Stone's gelatin agar.

In vivo tests have also been used. Dolman (1940) suggested the intraperitoneal injection of kittens with a sterile filtrate

containing the enterotoxic substance. Kitten feeding tests were used by Minett (1938), Ramsey and Tracy (1931), and Tanner and Ramsey (1932). Feeding tests on monkeys have been used by Dack, Bowman and Harger (1935), Kelly and Dack (1936), Jordan and Burrows (1934), Shaughnessy and Grubb (1936), Tanner and Ramsey (1932), and Davison, Dack, and Cary (1938). Other workers have used human volunteers (Dolman, 1934; Kelly and Dack, 1936; and Minett, 1938).

Various foods have been observed to be the cause of food poisoning outbreaks, but milk and milk products have been found to be the most common source of these infections. Foods involved have been cake, custard-filled and cream-filled bakery goods (Dack, Cary, Woolpert, Wiggers, 1930; Bransfield, 1937; Jordan, 1931; McBurney, 1933; Jordan and Burrows, 1934; Corpening and Foxhall, 1935); raw milk, (Barber, 1914; Ramsey and Tracy, 1931; Shaughnessy and Grubb, 1936; Crabtree and Litterer, 1934); cheese, (Jordan, 1930); chicken gravy (Jordan, 1931); chicken salad, (Hayes, 1935); tongue sandwiches (Dack, Bowman and Harger, 1935); oysters (Majors, Scherago and Weaver, 1938).

MATERIALS AND METHODS

Source of Organisms

All organisms studied were taken from milk samples sent to the bacteriology laboratory of Kansas State College for detection of streptococcic mastitis. The first half of the collection

of cultures studied was obtained by selecting hemolytic colonies from dilution plates poured with sheep blood agar. The remainder of the collection was obtained by streaking a loopful of the milk sample on (1) phenol red mannitol agar, (2) bromthymol blue agar, and (3) sheep blood agar. Typical colonies from these plates were isolated on a slant of stock infusion agar and the rest of the tests were run from these stocks. The cultures obtained in this manner were kept at room temperature and transplanted every month.

After the organisms were obtained in pure culture they were placed on the following media to differentiate between those which are usually considered capable of causing food poisoning and those which are not considered food poisoning types.

Media

Stock Agar and Broth. The medium used as a stock agar and the basal medium in the fermentation reactions was a meat infusion broth and agar. Five pounds of ground beef were infused with five liters of tap water in flowing steam for one hour. This was filtered through thick filter paper. Proteose peptone, two percent, and sodium chloride, 0.5 percent, were added and the pH adjusted to 7.8. After autoclaving for 15 minutes at 17 pounds, the medium was filtered through thick filter paper. The broth was stored in bottles and sterilized at 17 pounds, for 25 minutes. Using this broth as a base, two

percent agar-agar was added for the stock agar, and sterilized at 17 pounds for 25 minutes.

Fermentation Media. The following fermentation media were used in the tests:

Beef infusion broth	880 ml
Horse serum, sterile	10 ml
Carbohydrate (10% aqueous solution)	100 ml
Bromcresol purple (1.6% alcoholic solution)	1 ml

The broth-indicator mixture was sterilized at 17 pounds for 20 minutes. The carbohydrate solutions were prepared at pH 7.0-7.4, sterilized for 15 minutes at 15 pounds, and added aseptically to the broth. After the addition of the horse serum, the medium was tubed in sterile test tubes. Aesculin was added to the beef infusion broth, minus serum and indicator, to a concentration of 1.0 percent.

Glucose Broth. A 10 percent glucose solution was prepared and sterilized at 15 pounds for 15 minutes. To 900 ml of the sterile meat infusion broth were added aseptically 100 ml of the sterile glucose solution. This was incubated over night to test the sterility.

Sodium Hippurate Broth. To beef infusion broth, sodium hippurate was added to a final concentration of one percent. This was tubed in 5 ml amounts and sterilized at 17 pounds for 20 minutes.

Proteose Lactose Agar. The proteose lactose agar was composed of the following:

Beef extract	3 gm
Proteose peptone	5 gm
Lactose	10 gm
Agar	15 gm

The ingredients were dissolved in one liter of distilled water. The pH was adjusted to 7.6-7.8 and the medium tubed. After sterilizing at 15 pounds for 15 minutes, the medium was slanted and cooled.

Sheep Blood Agar. Five percent sterile whole sheep blood was added to melted meat infusion agar at 45°C. and poured into sterile Petri dishes.

Phenol Red Mannitol Agar, Bromthymol Blue Agar, and Crystal Violet Agar. The media of Chapman, Lieb, and Curcio (1937) as prepared by Difco was used.

Human Blood Plasma. Sterile blood was collected from the arm vein of a volunteer and one percent sodium citrated added to a concentration to prevent coagulation. The blood was centrifuged and the serum removed. The serum was tubed aseptically in 0.5 ml amounts in sterile Kahn tubes.

Stone's Gelatin Agar. The media as prepared by Difco was used in this test.

Technic of the Tests

Fermentations. A tube of beef infusion broth was inoculated from the stock culture and incubated for 48 hours at 37° C.

As the inoculum for each fermentation tube, 0.2 ml of this 48 hour broth culture was used. The carbohydrates used were: sucrose, salicin, inulin, mannitol, raffinose, sorbitol, aesculin, trehalose and lactose. The tubes were incubated for five days at 37° C. Fermentation of all carbohydrates was shown by the change of the indicator from purple to yellow. Fermentation of aesculin was tested by dropping a few drops of a one percent ferric citrate solution into the tube. Darkening of the media indicated fermentation.

Morphology in Glucose Broth. Five ml amounts of the media were inoculated with a loop of growth from the stock cultures. After incubation for five days at 37° C., a slide of the growth was made and stained with methylene blue to study the morphological characteristics.

Sodium Hippurate Fermentation. A tube of the media was inoculated with 0.2 ml of a 48 hour broth culture. This was incubated with an uninoculated control at 37° C. for five days. One ml of the uninoculated control tube was titrated with 12 percent ferric chloride, containing 2.5 ml concentrated hydrochloric acid per liter. The endpoint was that point at which all protein and hippurate precipitates are completely dissolved. This volume of the ferric chloride solution was then added to one ml of the media in each of the inoculated tubes. After a thorough shaking, a heavy, flocculent precipitate indicated a positive reaction, while a clear solution a negative reaction.

Pigment Production, Hemolysis Production, Fermentation of Mannitol, Growth of Bromthymol Blue Agar, Color of Growth on Crystal Violet Agar. The technic and criteria of Chapman, Lieb, and Curcio (1937) were used in these tests.

Coagulase Test. Five tenths of a ml of sterile human plasma were inoculated with the growth from a proteose lactose agar slant. The cultures were placed in the incubator at 37° C. The first reading was made after three hours, and the second after the cultures had been left over night in the incubator. If there was no apparent clot, the tubes were tilted to a horizontal position and examined for a jelly-like mass which could be seen rising slightly above the surface of the fluid, or as an opaque disc. Any of these effects were considered positive.

Liquefaction of Gelatin. The technic and criteria as given in the sixth edition (1939) of the Difco Manual were used.

EXPERIMENTAL RESULTS

Table 2 gives the results obtained with the organisms in these experiments. The tests used were pigment production (P), hemolysis production (H), coagulase production (C), luxuriance of growth on bromthymol blue agar (B), fermentation of mannitol on phenol red mannitol agar (M), color of growth on crystal violet agar (V), and liquefaction of Stone's gelatin agar (S). The last column in Table 2 gives the number of different series

of fermentation reactions in the broth media of that particular group. For example, line one, Table 2, could be divided into several reactions.

Two of the organisms listed in line one of Table 2 gave reactions as listed in column I, Table 1, two as listed in column II, two as listed in column III, and one as listed in column IV. The other reactions listed in Table 2 can be divided in this same manner.

Table 1. Fermentation reactions of organisms positive to PHCBMVS (with ++ Stone).

Carbohydrates	I	II	III	IV
Sucrose	-	-	+	-
Salicin	-	-	-	-
Inulin	-	-	-	-
Mannitol	+	+	+	-
Raffinose	-	-	-	-
Sorbitol	-	-	-	-
Aesculin	+	+	+	+
Trehalose	+	+	+	+
Lactose	+	+	+	-
Sodium Hippurate	+	+	+	+

+ indicates a positive reaction.
- indicates a negative reaction.

Table 2. Summary of reactions on the various media used.

P	H	C	B	M	V	S	No. of strains	% of strains	No. of milk samples	% of milk samples	No. different reactions
+	+	+	+	+	+	++	7	3.9	6	3.7	4
+	+	+	+	+	+	+	15	8.3	12	7.4	7
+	+	+	+	+	+	-	25	13.9	21	13.2	14
+	-	+	+	+	+	+	1	0.6	1	0.6	1
+	-	-	+	+	+	+	3	1.6	2	1.2	3
+	-	+	-	+	+	-	1	0.6	1	0.6	1
+	-	+	-	+	-	+	1	0.6	1	0.6	1
+	-	-	+	-	+	+	6	3.3	6	3.7	5
+	-	-	+	+	+	-	4	2.3	4	2.5	4
+	-	-	+	-	+	-	4	2.3	4	2.5	4
+	-	-	+	-	-	+	4	2.3	4	2.5	3
+	-	-	+	+	-	-	3	1.6	3	1.9	3
+	-	-	-	+	+	-	1	0.6	1	0.6	1
+	-	-	-	-	-	+	1	0.6	1	0.6	1
+	-	-	-	-	-	-	1	0.6	1	0.6	1
-	-	-	+	+	+	+	4	2.3	3	1.9	4
-	-	-	+	+	+	-	4	2.3	4	2.5	3
-	-	-	+	+	-	+	2	1.2	2	1.2	2
-	-	-	+	-	+	+	4	2.3	4	2.5	3
-	-	-	-	+	+	+	2	1.1	2	1.2	2
-	-	+	+	+	-	-	1	0.6	1	0.6	1
-	-	+	-	-	+	+	1	0.6	1	0.6	1
-	-	+	-	-	+	-	1	0.6	1	0.6	1
-	-	-	+	-	+	-	3	1.6	3	1.9	3

Table 2 (Continued)

P	H	C	B	M	V	S	No. of strains	% of strains	No. of milk samples	% of milk samples	No. different reactions
-	-	-	+	-	-	+	5	2.8	5	3.1	5
-	-	-	+	-	-	-	2	1.1	2	1.2	2
-	-	-	-	-	-	+	1	0.6	1	0.6	1
-	-	-	-	-	+	-	3	1.6	2	1.2	3
-	-	-	-	+	-	-	1	0.6	1	0.6	1
-	-	-	-	+	+	+	2	1.1	1	0.6	2
+	+	+	+	+	-	+	4	2.3	3	1.9	3
+	+	+	+	+	-	-	6	3.3	5	3.1	5
+	+	+	+	-	-	-	3	1.6	1	0.6	2
+	+	+	+	-	+	-	2	1.1	2	1.2	1
+	+	+	-	+	+	-	4	2.3	4	2.5	2
+	+	+	-	-	+	-	1	0.6	1	0.6	1
-	+	+	+	+	+	+	4	2.3	4	2.5	4
-	+	+	+	+	+	-	8	4.4	5	3.1	4
-	+	+	+	+	-	-	2	1.1	2	1.2	2
-	+	+	+	+	-	+	3	1.6	3	1.9	3
-	+	+	-	+	+	+	2	1.1	2	1.2	2
-	+	+	+	-	+	+	2	1.1	2	1.2	1
-	+	+	+	-	+	-	2	1.1	2	1.2	1
-	+	+	+	-	-	-	1	0.6	1	0.6	1
-	+	+	+	-	-	+	1	0.6	1	0.6	1
-	+	+	-	-	-	+	1	0.6	1	0.6	1
+	+	-	+	-	-	-	2	1.1	2	1.2	2
+	+	-	+	-	+	-	1	0.6	1	0.6	1
+	+	-	+	-	+	+	1	0.6	1	0.6	1

Table 2 (Continued)

P	H	C	B	M	V	S	No. of strains	% of strains	No. of milk samples	% of milk samples	No. different reactions
+	+	-	+	+	-	-	1	0.6	1	0.6	1
+	+	-	+	+	+	+	4	2.4	3	1.9	4
+	+	-	+	+	+	-	4	2.4	3	1.9	4
-	+	-	+	+	+	-	3	1.6	3	1.9	3
-	+	-	+	+	-	+	1	0.6	1	0.6	1
-	+	-	+	+	-	-	1	0.6	1	0.6	1
-	+	-	+	-	+	+	-	0.6	1	0.6	1
-	+	-	-	+	+	-	2	1.1	2	1.2	1
-	+	-	-	-	-	+	1	0.6	1	0.6	1
+	+	+	-	-	+	+	-	0.6	1	0.6	1

Twenty-two of the strains (12.2 percent) representing 18 milk samples (11.2 percent) gave positive reactions on all the media, indicating that they were probable food poisoning staphylococci. Chapman, Lieb and Curcio (1937) have suggested that the Stone reaction be applied only to variants which react positively to all the tests mentioned. By the term "variant" they refer to those organisms that at one time gave positive reactions to all the tests, but upon re-examination gave varying results. By following their suggestion, and ignoring the Stone reaction, there would be 25 additional strains (representing 21 milk samples) that are probable food poisoning varieties. Including these with the first figures in this study, there would be 47 strains (26.1 percent) representing 37 milk samples (25.4 percent) that give indication of being probable food poisoning staphylococci.

All organisms giving negative hemolysis could be classed as degenerates (see discussion), since that is the first reaction that is thought to be lost. Sixty-five organisms give negative hemolysis tests with various reactions in other tests. These 65 organisms are, probably, degenerates of probable food poisoning strains.

When checking the results of these experiments, it was noticed that 45 percent of those organisms giving positive PECVBMS reactions had been isolated from milk samples that had long-chain streptococci.

It was also noticed that 55.7 percent of the strains gave

a positive mannitol reaction in the tube fermentation while 69.7 percent gave positive reactions on the phenol red mannitol agar plates. Six and two tenths percent of the strains were positive in the tube, and negative on the plate, while 24.3 percent gave positive results on the plate and not on the tube. Discussion of this point will be presented later.

DISCUSSION

Chapman, Lieb and Curcio (1937) held that pathogenic staphylococci give positive reactions on the seven tests here used and Chapman[1] stated:

> When such pathogenic staphylococci are present in large numbers and in almost pure culture, and the findings are supported by epidemiological data, it is probable that they are of the food poisoning type. When no pathogenic type staphylococci are found in suspected food, food poisoning staphylococci can almost certainly be excluded.

Chapman, Lieb and Curcio (1937) further stated that the loss of any of these properties indicates that a strain is degenerating from the food poisoning type into a non-food poisoning type. This question now arises: Does this degeneration of biochemical characteristics also denote a degeneration in the ability to produce enterotoxin? Hemolysis appears to be the first biochemical characteristic to be lost, and thus is the best indicator of the degeneration of a strain.

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1 Personal communication from Dr. G. H. Chapman.

These tests are based upon a supposed relationship between cultural properties and toxin production. Since there may be no such relationship, the identification of staphylococci of the food poisoning variety must rest on other tests. According to Chapman[1]:

> The main objection to the use of in vitro tests in studies of pathogenic properties is based on a firmly established belief that they can never replace tests based on pathogenic effects on animals, it being assumed that pathogenicity is a mysterious vital complex which is different from any biochemical property of a culture. The assumption is made that pathogenic staphylococci produce "toxin" constantly under assumed optimum conditions and that non-pathogenic types fail to do so. Too little is known about the production and properties of staphylococcus toxins to permit this conclusion without reservation.

Data presented by Chapman, Lieb and Curcio (1937) indicate that strains of staphylococci thought to be the cause of bovine mastitis in cows give positive PhCVBMS reactions. Shaughnessy and Grubb (1937) stated that

> It has been our experience to find outbreaks of milk poisoning only where one or more of the cows producing the milk have a staphylococcus mastitis.

But neither these groups of workers, nor others working with staphylococci of bovine origin have mentioned any connection between a streptococcus mastitis and the presence of probable food poisoning staphylococci. Shaughnessy and Grubb (1937) concluded that

> If a large number of staphylococci in practically pure culture are found in the incriminated milk, and other microscopic and chemical tests indicate that one or more of the cows supplying the milk has mastitis; and the epidemiological evidence and clinical symptoms point to milk-borne staphylococcus poisoning, it is believed

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1 Personal communication from Dr. G. H. Chapman.

that this presumptive evidence is strong enough to warrant the diagnosis of a staphylococcus milk poisoning and the elimination of the infected cows from the herd.

At the present time animals may be eliminated from a herd when long-chain streptococci can be demonstrated in the milk. From the results of these experiments, eight of the 17 samples of this milk would be condemned. As far as the staphylococci in the milk are concerned, one group of cows would be considered just as dangerous to health as the other.

The present results and comparisons of mannitol fermentation do not agree with those of other investigators (Plastridge, Anderson, Williams and Weirether, 1939). The results they obtained with phenol red mannitol agar were similar to those obtained with mannitol broth, while the present reactions differed considerably.

It is interesting to note the lack of correlation between the fermentation tests as performed with broth media and the results obtained from the use of the PHCBMVS reactions as shown in Tables 3, 4, and 5.

The data in Table 3 were obtained by dividing the organisms that gave positive PHCBMVS (with ++ Stone) into their reactions on the broth fermentation media. The same thing was done for the organisms with positive PHCBMVS (with + Stone) to obtain the data for Table 4.

For Table 5, the organisms with fermentation reactions similar to those in column IV, Table 4, are divided into the different reactions on Chapman, Lieb, and Curcio's tests.

From Tables 3, 4 and 5 it can be seen that there is very little correlation between the fermentation tests performed in broth media and the tests conducted according to Chapman, Lieb and Curcio (1937).

Table 3. Fermentation reactions of organisms positive to PHCBMVS (with ++ Stone).

Carbohydrates	I	II	III	IV
Sucrose	+	-	+	-
Salicin	-	-	-	-
Inulin	-	-	-	-
Mannitol	+	+	+	+
Raffinose	-	-	-	-
Sorbitol	-	-	-	-
Aesculin	+	+	+	+
Trehalose	+	+	+	+
Lactose	+	+	+	-
Sodium hippura te	+	+	+	+
No. of organism	2	2	2	1

Table 4. Fermentation reactions of organisms positive to PHCBMVS (with + Stone reaction).

Sucrose	+	+	+	+	-	-	+
Salicin	-	-	-	-	+	-	-
Inulin	+	+	+	-	-	-	-
Mannitol	+	+	+	+	+	+	+
Raffinose	-	+	+	-	-	-	-
Sorbitol	-	-	-	-	-	-	-
Aesculin	-	-	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	+
Sodium Hippurate	+	+	+	+	+	+	+
No. of organisms	1	1	2	7	1	2	1

Table 5. Classification of organisms with fermentation-reactions similar to those listed in column 4, Table 3.

P	H	C	B	M	V	S	No.
+	+	+	+	+	-	-	2
-	+	+	+	+	-	-	1
+	+	+	+	+	+	+	3
+	+	+	+	+	+	++	2
+	+	+	+	+	-	+	1
+	+	+	+	-	-	-	1
+	+	+	+	+	+	-	4
-	+	+	+	+	+	-	4
-	-	+	+	+	-	-	1
+	+	-	+	+	+	+	1
-	+	-	+	+	-	+	1
-	+	+	+	+	-	+	1
-	-	+	+	+	+	+	1
+	-	+	-	+	+	-	1
+	+	+	-	+	+	-	3

CONCLUSIONS

Eleven and one-tenths percent of the 161 milk samples examined in this investigation gave evidence of containing probable food poisoning staphylococci. With the exclusion of the Stone Reaction, 26.4 percent of the milk samples contained probable food poisoning staphylococci.

There seemed to be very little correlation between the fermentation reactions, as a whole, and the tests outlined by Chapman, Lieb and Curcio (1937).

The varying results obtained in the fermentation of mannitol in broth and the agar plate would indicate that more work is needed before definite comparisons of fermentation reactions as described in the literature can be made.

Forty-seven percent of the milk samples containing probable food poisoning staphylococci also contained long-chain streptococci. It would seem to be just as necessary to examine milk for staphylococci, and to condemn it for use as human food on the presence of a large number of staphylococci, as on the presence of long-chain streptococci.

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